Hepatitis E: prospects for immunoprophylaxis

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Hepatitis E is the most important cause of clinical acute hepatitis among adults in much of Asia and the second most important cause in the Middle East and North Africa. Antibody to hepatitis E virus (HEV) is more prevalent than antibody to hepatitis C virus (HCV) in industrialized countries, although clinical hepatitis E is rare. Epidemiologic evidence suggests zoonotic spread of HEV, as well as human-to-human spread, usually via water. Previous studies have demonstrated the feasibility of passive and active immunoprophylaxis in animal models (non-human primates). Based on those studies, hepatitis E vaccines have been developed. The most extensively tested are expressed from baculovirus in insect cells. They are highly immunogenic and protect macaques from hepatitis E caused by heterologous as well as homologous challenge virus. Based on these studies, lots of hepatitis E vaccine suitable for clinical evaluation have been prepared. These have passed all preclinical safety and efficacy trials. In preliminary studies, the vaccine was safe and immunogenic in humans; it is currently being tested for efficacy in Nepal.

Why a hepatitis E vaccine ?

Clinical hepatitis E is rarely diagnosed in industrialized countries of Europe or North America. However, it is the single most important cause of acute clinical hepatitis among adults in much of Southeast and Central Asia and the second most important cause of such hepatitis in parts of the Middle East and North Africa [1–3]. Furthermore, as is the case for hepatitis A, the vast majority of infections (detected by serological means) go undiagnosed, probably because they are not apparent or are subclinical infections. Thus, an estimated 5 billion of the world's 6 billion population have been infected with hepatitis A virus (HAV) and perhaps 2 billion of the world's population have been infected with hepatitis E virus (HEV). Although, again as with HAV, none of these HEV infections progresses to chronicity, they do pose a risk of serious disease, especially during water-borne epidemics and especially to pregnant women, in whom hepatitis E may have a mortality of 20%. Furthermore, unlike the other hepatitis viruses, HEV appears to infect many species of wild and domesticated animals, including rats, mice, swine, sheep and cattle [4]. These species, often living in close contact with man, may serve as reservoirs for zoonotic spread from animals to humans, although this has not been proven. Finally, hepatitis E is a potential threat to travelers who visit regions where the virus is endemic, and most reported cases of hepatitis E in industrialized countries have resulted from such infections in travelers who returned home during the incubation period and became ill shortly thereafter [5]. For these reasons, an effective and relatively inexpensive hepatitis E vaccine would be a useful addition to our armamentarium of immunoprophylactic agents.

Prospects for control

Again, as is the case with HAV, improved sanitation is the first line of defense in the control of HEV. In fact, the improved public health resulting from updating of sewage and water systems in industrialized countries in past decades has probably contributed significantly to the low level of endemic spread of HEV and the absence of epidemics of disease in these countries when compared to developing countries. In contrast, annual water-borne epidemics, coinciding with monsoon seasons or spring thaws, occur in some countries, whereas in others, such as Egypt, infection appears to be highly endemic in the absence of defined epidemics [6]. It would therefore be useful to have immunoprophylactic measures, such as passive immunoprophylaxis (immunoglobulin) and active immunoprophylaxis (vaccine) for widespread use in endemic countries and targeted use in industrialized countries.

Animal models

Although several species of non-primates have been shown to be susceptible to HEV infection by seroepidemiology or direct transmission, none of these has been useful for vaccine development. Instead, non-human primates, especially rhesus and cynomolgus monkeys and chimpanzees, have been the most useful surrogates of man for testing vaccines.

Passive immunoprophylaxis

Evidence that prior exposure to HEV, as measured by pre-existing anti-HEV, is associated with protection against subsequent exposure to the virus has come from

epidemiologic studies of water-borne epidemics of HEV in Pakistan [7,8]. In these epidemics, which occurred among members of the military, the clinical attack rate (as measured by hospitalization) among the cohort of 63 individuals who lacked anti-HEV at the beginning of the epidemics was 37%. In contrast, not a single one of the 46 individuals who were positive for IgG anti-HEV at the beginning of the epidemics was hospitalized.

That this protection was probably antibody-mediated comes from other epidemiologic and laboratory-based studies. Although none of the immunoprophylaxis trials of normal immune globulin in countries where HEV is endemic has demonstrated statistically significant protection, even when the globulin was manufactured within the country, some have provided suggestive evidence. The failure to demonstrate significant protection in such studies is probably the result of the relatively low titers of anti-HEV found in globulin preparations, coupled with the fact that the immune globulin was usually administered late in the epidemic. However, direct demonstration of the protective efficacy of anti-HEV has come from studies in which convalescent plasma or serum obtained from naturally infected patients or experimentally infected non-human primates, when infused into naive non-human primates, has protected them against hepatitis (but not necessarily infection). In contrast, serum or plasma from non-infected humans or non-human primates, when infused into naive animals, has failed to protect against subsequent challenge with virulent HEV [9,10]. Thus, if an immunoglobulin preparation with a sufficiently high titer of anti-HEV could be prepared, it probably would be efficacious in preventing HEV when administered to those who are exposed. One potential source of such 'hepatitis E immune globulin' might be individuals who have been repeatedly vaccinated with hepatitis E vaccine, once it has become available. Alternatively, recently described monoclonal antibodies that can neutralize HEV *in vitro* may be especially useful for this purpose [11]. These monoclonal antibodies, which were derived from a combinatorial library obtained from the bone marrow of an experimentally infected chimpanzee, are virtually identical to globulins of human origin. They should behave in humans in a manner identical to human globulins, since human globulins have a half life in chimpanzees that is identical to the half life of such globulins in humans.

Active Immunoprophylaxis

Candidate HEV proteins for vaccine development

Among the proteins of HEV, those encoded by open reading frame (ORF) 1 are not the best candidates, because they are non-structural and therefore not accessible to antibody; a vaccine constructed from ORF1 proteins would have to protect through cellular immune mechanisms. Furthermore, although at least some of the ORF1-encoded proteins are immunogenic, there are no data on their ability to elicit protection [12]. It is not known whether the protein encoded by ORF3 is structural or non-structural. It is immunogenic, however antibody to it is short-lived and relatively genotype-specific [13]. Finally, antibody to the ORF3 protein does not neutralize the virus [14]. This leaves the protein encoded by ORF2. The ORF2 protein is believed to be the capsid protein of the virus. Its sequence is highly conserved and antibody to it is long-lived and cross-reactive among diverse strains [13]. Finally, antibody to the ORF2 protein neutralizes HEV *in vitro* and protects non-human primates against HEV following challenge with virulent virus [9,11,14,15]. Thus, the capsid protein, encoded by ORF2, is the best candidate for vaccine development.

Table 1. Hepatitis E virus (HEV) open reading frame (ORF) 2 antigenic peptides expressed in E. coli (as GST fusion protein)

Reference	HEV (origin)	Designation	Amino acids N'	Amino acids C'
16	China	ORF2	1	660
17,18	Burma	trpE-C2	221	660*
19	Burma	SG3	328	654†
20–22	China	ORF2.1	394	660
23	Mexico, Burma	3.2	612	654†

* Tested as vaccine.
† In commercial diagnostic tests.

Recombinant proteins expressed from the HEV ORF2

Since HEV grows poorly if at all in cell culture, proteins for diagnostic use and vaccine development have been recombinantly expressed in a variety of systems but principally from *Escherichia coli* as fusion proteins or in insect cells from baculovirus vectors. These are depicted in Tables 1 and 2. Although the first candidate HEV vaccine was expressed in *E. coli*, the proteins derived from bacteria have been used principally as antigens for diagnostic purposes [16–22]. Interestingly, the largest and smallest of these have been superseded by proteins of intermediate size because of superior sensitivity for detecting anti-HEV in ELISA and Western blot assays [13]. Their increased sensitivity appears to result from their ability to fold into native configurations that reveal conformational epitopes or that unmask linear epitopes [16].

Similarly, proteins expressed in insect cells have been useful antigens for detecting anti-HEV but full-length proteins have been either insoluble or relatively insensitive for serologic use [23–31]. However, proteins expressed in such systems are usually post-translationally processed in several ways. First, at least some of the proteins are larger than their amino acid sequence would predict: proteins with calculated molecular masses of 50 and 53 kDa were actually 53.8 and 56.1 kDa, respectively, when measured by mass spectroscopy [26]. It is not clear whether this is the result of glycosylation, myristylation or some other form of protein modification [32,33]. Two sites for N-linked glycosylation and seven sites for myristylation are present in these proteins [26]. Second, proteinases that are

Table 2. Hepatitis E virus (HEV) open reading frame (ORF) 2 antigenic peptides expressed in insect cells (from baculovirus vector)

Reference	HEV (origin)	kDa (mol. mass)	Amino acids N'	C'	VLP
24,25	Burma	72	1	660	?
26	Pakistan	63	112	660	?
27–29	Burma	62 (56 549)	112	636	±*
9,26,34	Pakistan	55 (56 144)	112	607	±*
26	Pakistan	53 (53 872)	112	578	†
30,31	Burma	50	112	?	†

* Candidate vaccine
† Assembles into virus-like particles

probably encoded by the baculovirus vector cleave the ORF2 protein between amino acids 111 and 112, thereby removing the hydrophobic signal sequence and rendering the protein more soluble [29,34]. Third, proteinases successively truncate the protein from the carboxy end, yielding a series of proteins of different sizes and with different characteristics. For example, proteins of approximately 55 kDa and larger are retained within the insect cell and, following cell lysis, they can be purified to a high degree of homogeneity as monomers [26,28,29]. In contrast, some proteins of approximately 53 kDa and smaller are secreted from insect cells and form virus-like particles (VLPs) that are smaller than the intact virion of HEV [26,30]. The VLPs derived from 50 kDa ORF2 proteins have been shown to be highly ordered icosahedrons with two-, three- and five-fold symmetry [31]. Indeed, VLPs have been reported in preparations of the larger proteins, but these are either formed inefficiently or are the products of smaller forms of the protein that exist in the preparations as minor populations [25,35]. However, the larger and smaller proteins differ in another important respect: those proteins described in Table 2 that are 55 kDa or larger contain an important neutralization epitope that exists approximately between amino acids 575 and 610 [11,36]. To date, this is the only bona fide neutralization site that has been identified.

Two of the recombinant proteins, a 62 kDa protein developed by McAtee *et al.* [28,29] at Gene Labs Inc., and a 55 kDa protein developed by Tsarev *et al.* [25,26] in the Hepatitis Viruses Section of the National Institute of Allergy and Infectious Diseases, have been studied more or less extensively in non-human primates as candidate vaccines [9,15]. Both efficiently protected cynomolgus or rhesus monkeys from hepatitis E following challenge with virulent HEV. Partly because of its stability when formulated as a vaccine, the 55 kDa protein was chosen for further clinical development.

Preclinical evaluation

The HEV ORF2 gene from the Sar-55 (Pakistan) strain of HEV was truncated to encode amino acids 112 through 660 and inserted into a baculovirus vector by standard methods. Insect cells (Sf-9) were infected with the recombinant baculovirus and the recombinant ORF2 protein was expressed in large quantities. It underwent further proteolytic truncation intracellularly by cleavage between amino acids 607 and 608. This doubly-truncated protein, with a molecular mass of 56 144, was highly purified and adjuvanted with alum. The candidate vaccine was administered intramuscularly to seronegative rhesus monkeys, which were then challenged intravenously with virulent HEV.

The vaccine was highly immunogenic: formulations of 50, 10, 2 and 0.4 µg all resulted in 100% seroconversion following a single dose of vaccine [37]. Following a second dose of vaccine, the geometric mean titers of anti-HEV were >1:1000 and all of the vaccinated animals were protected against hepatitis E (but not necessarily infection) following intravenous challenge with $10^{3.0}$ to $10^{4.5}$ monkey 50% infectious doses (MID_{50}). Failure to protect against infection may have been the result of using such a massive challenge dose, a necessity to assure that all of the control animals developed hepatitis, since low doses of challenge virus result in infection but not necessarily hepatitis [38]. In similar studies, the vaccine protected against not only challenge with the homologous (SAR-55) virus but also challenge with a heterologous Mexican (MEX-14) strain of HEV [37]. When challenge was delayed for 6 or 12 months following completion of vaccination, protection was also achieved, although one of four monkeys, which had lost detectable anti-HEV by the time of challenge, was not protected (Purcell *et al.*, unpublished data; [39]). Finally, attempts to demonstrate post-exposure vaccination, by challenging intravenously with HEV and then initiating vaccination within 48 h, was unsuccessful, perhaps because of the large challenge dose of virus [37].

Based upon these vaccination studies, SmithKline Beecham Biologicals (now Glaxo SmithKline: GSK), Rixensart, Belgium, sponsored the preparation of hepatitis E vaccine suitable for clinical evaluation. This was manufactured by DynCorp (now Novavax), Rockville, Md., USA. Following tests of safety and purity by GSK, the clinical lot was evaluated in rhesus monkeys for immunogenicity and efficacy. Three vaccination regimens (two doses of 1 µg, two doses of 10 µg, one dose of 10 µg) were compared with a placebo (the GSK Havrix hepatitis A vaccine; Table 3). All three vaccination regimens were immunogenic, but two doses of vaccine were necessary for peak titers. One µg of vaccine was as immunogenic as 10 µg. Geometric mean titers of approximately 1:9000 were achieved following two doses of vaccine, regardless of formulation. In contrast, a single 10 µg dose produced anti-HEV titers only approximately one-sixth as high.

Vaccinated and control monkeys were challenged with $10^{4.0}$ MID_{50} of the homologous virus (SAR-55) or one of two heterologous viruses; a Mexican strain (MEX-14) or a US strain (US-2). Preliminary virologic evaluation revealed complete protection against viremia in most vaccinated animals and markedly diminished viremia in the remainder when compared to unvaccinated animals (Purcell *et al.*, unpublished data). Thus, the candidate vaccine was highly

Table 3. Protection of macaques against hepatitis E by active immunoprophylaxis

Vaccine	Pre-challenge anti-HEV Approximate GMT	WHO units*	Post-challenge: ration of GM peak/pre ALT following challenge with HEV Pakistan	Mexico	USA
2×1 mg	29 000	483	1.2	1.2	1.3
2×10 mg	20 000	483	1.1	1.1	1.1
1×10 mg	3600	60	1.4	1.5	2.0
Havrix	Neg.	0	8.3	6.0	3.2

*WHO standard (convalescent serum): 100 units.
Challenge dose: $10^{4.5}$ MID_{50}, iv
HEV, hepatitis E virus; ALT, alanine aminotransferase.

immunogenic and efficacious in rhesus monkeys and therefore suitable for clinical evaluation in humans.

Two doses of vaccine (1 or 10 μg) were necessary for complete protection against hepatitis E following challenge of the vaccinated monkeys. In contrast, a single dose of 10 μg of vaccine afforded only partial protection when peak liver enzyme values were compared with the placebo-vaccinated monkeys following challenge (Table 3).

Clinical evaluation

Clinical evaluation of candidate HEV vaccine was designed and performed by the staff of the Walter Reed Army Institute of Research (WRAIR). Phase I safety trials were performed in 88 healthy adult volunteers in the USA. Three doses (at 0, 1 and 6 months) of four formulations (1, 5, 20 and 40 μg) were compared [40]. All formulations were well tolerated and immunogenic, especially following the third dose of vaccine. Formulations of 5 and 20 μg were selected for further development.

The second phase of safety and immunogenicity trials was carried out in Nepal, where hepatitis E is highly endemic and occurs as water-borne epidemics following flooding caused by the annual monsoons. In these studies 44 healthy adult seronegative volunteers were immunized with 5 or 20 μg of vaccine at intervals of 0, 1 and 6 months. Again, the vaccines were well tolerated and immunogenic, causing seroconversion in 100% of vaccinated individuals following the third dose of either formulation [41].

Based upon these encouraging results, a preliminary combined immunogenicity and efficacy trial has been initiated in Nepal by WRAIR. The vaccine will be administered in formulations of 5 and 20 μg and the results compared with a placebo control vaccine. The vaccines and placebo will be administered on a schedule of 0, 1 and 12 months, and vaccinees will be followed for 2 years. This will permit evaluation of two doses of vaccine during the first year of observation and evaluation of three doses during the second year.

The future

A number of questions remain to be answered about the immunoprophylaxis of hepatitis E. As regards passive immunoprophylaxis, antibody to the capsid protein of HEV is cross-reactive among different genotypes of the virus but it is not clear whether antibody-mediated protection is quantitatively the same with all genotypes. In particular, if monoclonal antibodies are used for passive immunoprophylaxis, it must be demonstrated that they are sufficiently cross-reactive to be efficacious where major genotypes overlap geographically.

As regards active immunoprophylaxis, antibody against the capsid protein is long-lasting, but the duration of antibody-mediated protection against hepatitis E remains to be determined. There is limited evidence suggesting that the detection of antibody to the HEV capsid protein correlates with protection but that, as such antibody falls below detectable levels, protection against clinical hepatitis may diminish. However, rhesus monkeys that were rechallenged with HEV up to 5 years after primary challenge with a heterologous strain were still immune to hepatitis E and, based on decay curves of the monkeys' anti-HEV titers, protection was estimated to last over 20 years [42]. Finally, the entire spectrum of genotypes of HEV has probably not yet been identified, and strains that are more divergent might emerge. Nevertheless, current knowledge about hepatitis E supports the view that the experimental vaccine currently under evaluation is a promising candidate for the control of hepatitis E.

Acknowledgments

Animal studies were performed at Bioqual Inc., under NIAID Intramural contract No. 1-AO-02733 (and previous contracts) and a CRADA with Glaxo SmithKline Biologicals, Rixensart, Belgium. I wish to thank Marisa St Claire and Max Shapiro for animal studies, Ronald Engle for serologic testing, Doris Wong for management and coordination, Robert Kuschner for sharing clinical data, Suzanne Emerson for reading of the manuscript and discussions and Janine Juliussen for secretarial and editorial assistance.

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